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The structure and proteomic analysis of byssus in *Pteria penguin*: Insights into byssus evolution and formation

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Highlights

- Highly aligned fiber core, cuticle with protein granules and porous adhesive plaque constitute the *Pteria penguin* byssus.
- Seven types of extracellular matrix proteins and related domains are detected in *Pteria penguin* byssus.
- Similar proteins involved in framework formation and immunity are present in byssus and shell fabrication.

Abstract

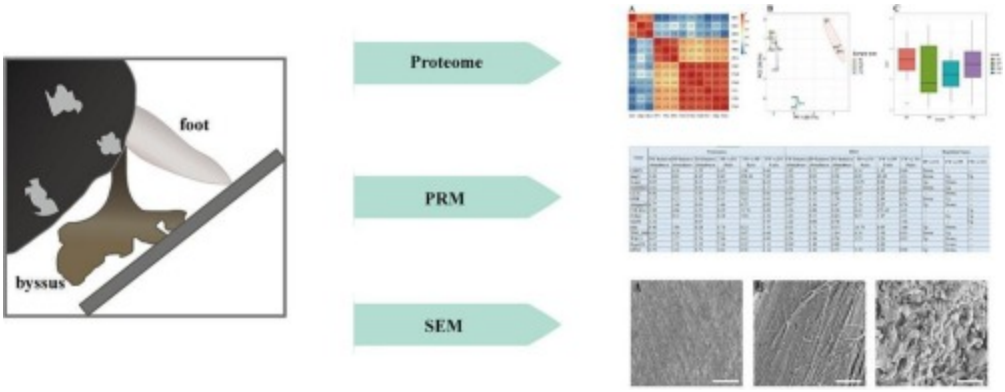
Byssus is a unique external structure in sessile bivalves and is critical for settlement and metamorphosis. However, little is known about the stout byssus in *Pteria penguin*. We

explored the byssus structure and proteins using scanning electron microscopy and proteomics, respectively. The results revealed that *P. penguin* byssus has a dense and highly aligned fiber inner core, and the outer cuticle contains protein granules embedded in the protein matrix. Proteomic analysis revealed 31 proteins in the byssus, among which 15 differentially expressed proteins were mainly enriched in the EGF/EGF-like and laminin EGF-like domains. Foot proteins were enriched in the EF-hand, immunoglobulin, and fibronectin domains. All these domains can participate in protein-protein and/or protein-metal interactions in the extracellular matrix (ECM), which, together with the seven types of ECM proteins detected in the byssus, supports the hypothesis that the byssus is derived from the ECM. We also found that *in vitro* acellular structures of the byssus and the shell shared commonalities in their formation processes. These results are useful for further understanding byssus evolution and the characterization of byssus-related proteins.

Significance

This manuscript investigates the structure and the origin of *Pteria penguin* byssus, given that byssus is vital to provide critical protection for reproduction and even against environmental stresses that affect survival. However, there is rare research on byssus protein composition. Hence, though scanning electron microscopy and proteomic analysis, we discovered that *P. penguin* byssus possesses the dense and highly aligned fiber inner core, and the outer cuticle has protein granules embedded in the protein matrix. Proteomic analysis showed that there were 31 proteins in the byssus, among which 15 proteins were mainly enriched in the EGF/EGF-like and laminin EGF-like domains. Foot proteins closely related to byssus formation were enriched in EF hand, immunoglobulin, and fibronectin domains. These domains are able to participate in protein-protein and/or protein-metal interactions in the extracellular matrix (ECM), which together with the seven types of ECM proteins detected in byssus support the hypothesis that byssus derive from the ECM. We also found *in vitro* acellular structures the byssus and the shell share commonalities in their formation processes. These results were useful for further understanding the byssus evolution and the characterization of the byssus-related proteins.

Graphical abstract



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Introduction

The byssus of bivalves, distinct from spiders and sea stars where the adhesive materials could separate from the organisms and deposited independently, is probably the most investigated bio-adhesion system [1,2]. It is composed of multiple byssal proteins secreted by the foot glands rooted in the foot of the bivalve and is vital to tether themselves in the habitat and survive environmental stress [3]. The byssus is a unique adhesive structure in marine bivalves composed of proteins [4,5], and the slow spread of the byssus serving as an anchor drives adaptive radiation in species that use it for hard substrate attachment [6]. In the larval stage, all bivalve species appear to be byssate, and the initial function of the byssus is to anchor itself to resist environmental impacts during metamorphosis [5]. With growth and development, the byssus can adhere to various substrates to facilitate feeding, locomotion, and survival [7] and plays a critical role in bivalve settlement and benthic life [5]. The byssus contributes to environmental adaptability and the occupation of niches, leading to ecological success [8,9]. However, the byssus is absent from adults such as hard clams [10], scallops [11], and all true oysters from Ostreidae [12] due to their life habits.

Highly heterogeneous and diverse adult byssus morphologies are present in marine bivalves. Multiple scattered and radical-distributed threads consist of the mussel byssus; there are, on average, 50 to 100 slender threads from each mussel, and the proximal region is bundled into a stem rooted in the foot, while the distal region ends in the attachment plaque [13,14]. The giant clam, *Tridacna maxima*, exhibits a silk-like brush byssus composed of hundreds of threads [15], whereas the long and thin byssus of fan shells (as many as 30,000) acts more like anchoring plant roots [1]. In scallops, the byssus consists of ribbons rather than threads [16,17]. The byssus structure is absent in ark clams, where threads are

fused into a sheet or plug-like structure, but no stem structure is observed [5,18]. The most unusual byssus is derived from the winged pearl oyster, *Pteria penguin*, in which all secreted byssus eventually merge into a strong single byssus [19,20], unlike mussels with well-defined byssal threads and attachment plaques. The byssus number, color, and diameter of the winged pearl oysters were also different from those described above [21]. This demonstrates the fundamental differences in the byssus between species and the necessity of investigating the molecular field to further understand the byssus and its evolution.

As an acellular and high-performance proteinaceous anchor, the demonstration of excellent underwater adhesion properties is not only due to muscle strength [22] but also to the ability of byssal proteins secreted by foot glands to rapidly self-assemble and function outside the body [23]. Previous studies have shown that the number and morphology of foot glands vary across species [[24], [25], [26], [27], [28]], yet the secreted byssal protein categories are homologous across species. Investigations of byssal proteins have mainly been performed on mussels; >10 byssal proteins have been identified, including 6 mussel foot proteins (Mfp-1-6), 3 pre-polymerized collagens (preCOLs: preCOL-D, preCOL-P, and preCOL-NG), 2 thread matrix proteins (TMP and PTMP-1), and polyphenol oxidase [4,29]. Three regions with completely distinct compositions and functions were integrated into a single cohesive byssus: the cuticle, core, and plaque [28]. The major coating protein, Mfp-1, is cross-linked by polyphenol oxidase during byssus cuticle formation, which confers insolubility and durability to the byssus [2,30]. Flexible fiber preCOLs with semi-crystalline arrays form the inner core [31,32], which has different mechanical properties owing to its distribution, structure, and hardness along the length of the byssus [33], whereas thread matrix proteins are involved in bridging the collagen fibers laterally [34]. The 3,4-dihydroxyphenylalanine (DOPA)-enriched proteins Mfp-2-6 function in plaque adhesion [4, [27], [28], [29]].

Mounting investigations have identified novel byssal proteins, indicating that byssal proteins have low homology and poor sequence conservation among different marine bivalves. Putative new foot proteins Mcfp-7p-14p have been identified in *Mytilus californianus*, of which Mcfp-10p contains three tandem repeat domains with no equivalent database homolog [27]. The three annotated scallop byssal proteins, Sbp-1, Sbp-3, and Sbp-7, shared 29%, 34%, and 28% sequence identity with the previously reported CD109 antigen, serine protease inhibitor dipetalogastin, and metalloproteinase inhibitor 2, respectively. However, the four novel Sbp components showed no significant matches [17]. Pearl oysters have completely different byssal proteins at the ultrastructural level [1]; however, research in this field remains fragmentary. Therefore, despite the remarkable significance of the byssus in marine bivalve evolution and development, limited molecular data and a lack of

protein comparisons across species have hindered our in-depth understanding of byssus evolution.

Bivalves have evolved a great variety of byssus, and their ability to use the byssus tether themselves to substrates underwater is central in their ecological and evolutionary success [35,36]. The byssus varies between species, among which the *P. penguin* byssus is unique in that all the byssus it secretes eventually fuse together to form a single, thick byssus (Fig. S1). However, little is known about its formation and evolutionary origins. In this study, we used scanning electron microscopy (SEM) to explore the byssus ultrastructure and a 4D label-free quantitative proteome to guide the investigation of protein composition within the *P. penguin* byssus and foot. These insights into the byssus could enrich the knowledge of the byssal protein composition of bivalves, enhance the understanding of mollusk evolution.

Section snippets

Sample collection

A total of 1000 individuals (shell length 6–8 cm) were transported to the Haichang aquaculture farm in Wenchang, Hainan Province, China. Samples with byssus were collected and cut using clean scissors. These samples were acclimated in a cement pond filled with aerated filtered seawater and fed with *Isochrysis galbana* (concentration: $2.5–3.0\times10^4$ cells mL^{-1}). Seawater was maintained at a temperature of 26±1 °C and salinity of 34±1. After a week, oysters cultured in different panel nets were …

Byssus structure

SEM observations revealed three regions of the *P. penguin* byssus: the cuticle structure on the surface, the highly aligned core, and the porous adhesive plaque. There was no clear separation between the byssus thread and the adhesive plaque, and the surface structure of the byssus thread (Fig. 1A, B) was identical to that of the adhesive plaque, which was also

consistent with the proximal and distal surface structures of the whole byssus thread. The surface and internal structures of the byssus …

Discussion

The byssus comprises a soft collagenous inner core surrounded by a hardened cured outer cuticle consisting of polyphenolic proteins [4]. However, many ultra-structurally distinct byssi have appeared throughout evolution. Apart from the typical triple-helix collagenous byssus of the model organism mussels, the byssus of *Pinna nobilis*, *Atrina pectinate*, and *Pinctada fucata* consist of globular protein arranged helically into fibers [1], whereas that of the giant clam, *T. maxima*, is constructed of…

Conclusion

This study employed proteomics to analyze byssus-related proteins in the byssus and foot of *P. penguin* and probed the ultrastructure of the byssus using SEM. The SEM observation of byssus led to the discovery of the dense, highly aligned fibrous core and a cuticle with granules embedded in a protein matrix, which is analogous to that of mussels. Based on proteomic analysis, we identified protein components (ECM and others) that may constitute the basic framework of the byssus, including…

CRediT authorship contribution statement

Yi Chen: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Hengda Chen:** Software, Investigation, Data curation. **Changqing Han:** Methodology, Investigation. **Huilong Ou:** Supervision, Project administration, Methodology, Funding acquisition, Data curation. **Xin Zhan:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.…

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.…

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